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Award Number: DAMD17-03-1-0557

TITLE: Identification of Intravasation Sites Using Magnetic Resonance Imaging

PRINCIPAL INVESTIGATOR: Jeffrey E. Segall, Ph.D.

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine
Bronx, NY 10461

REPORT DATE: August 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-08-2005	Final	15 Jul 03 – 14 Jul 05

4. TITLE AND SUBTITLE
Identification of Intravasation Sites Using Magnetic Resonance Imaging

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0557

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jeffrey E. Segall, Ph.D.

E-Mail: segall@aecom.yu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albert Einstein College of Medicine
Bronx, NY 10461

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

No abstract provided.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Metastasis is the major cause of death of breast cancer patients. An important step in metastasis is the intravasation of tumor cells, and we hypothesize that leakage of serum components from blood vessels can stimulate intravasation and metastasis. Alternatively, we have also found that macrophages in the tumor can enhance metastasis. Thus assays that would evaluate blood vessel leakiness and macrophage density over the entire tumor could improve our ability to identify metastatic tumors. Magnetic resonance imaging is well established as a method for evaluating blood vessel properties in patients, and probes have also been developed to identify macrophages (due to their high phagocytic ability). We proposed to study several mouse models of metastatic breast cancer in order to identify MRI imaging methods that might be useful for identifying metastatic tumors.

BODY

Task 1. Register 3D tumor images as determined by intravital imaging of GFP-labeled tumor cells with MRI images utilizing tumor structure and blood vessel pattern as determined by unenhanced and Gd/SPIO-enhanced imaging (Months 1 - 4):

a. *Determine time and parameters required for stable MRI imaging of tumors at low resolution (200 um x 200 um x 200 um) and selected areas at high resolution (100 um x 100 um x 100 um) for registration with light microscope imaging – 5 mice.*
b. *Evaluate low and high molecular weight gadolinium-based probes as markers for blood vessels, blood vessel permeability, and macrophages – 5 mice.*
c. *Evaluate small particle iron oxide probes with differing surface coatings as markers for blood vessels, blood vessel permeability, and macrophages – 5 mice.*

Our initial studies utilized tail vein catheterization and injection of the gadolinium-based MRI agent Magnevist, as had been published by other groups. Although peripheral vein catheterization is relatively straightforward for rats or larger mammals, we discovered that expertise in tail vein catheterization in SCID or C57-Bl mice is extremely challenging. We were unable to get rapid entry of the label into blood vessels, and hypothesize that in most cases the material was injected into the tail connective tissue next to the vein. The dye then slowly entered the circulation probably through a combination of diffusion into the tail vein and traveling the lymphatic system. However, we were able to image tumors after the Magnevist entered the circulation (Figure 1). This enabled us to determine the appropriate parameters for imaging the tumor using the 9.4 T magnet. Although dynamic changes in Magnevist could be measured, the slow increase and decrease in circulation levels indicated that the entry was not in a bolus as required for quantitative permeability measurements.

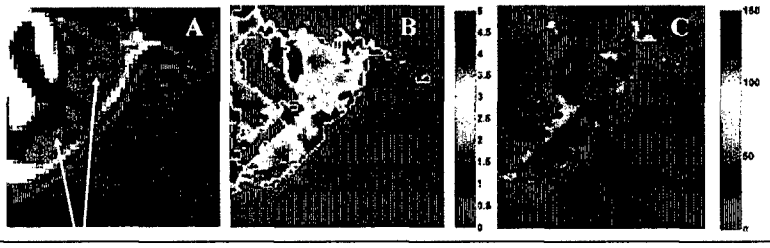

Figure 1: Gadolinium enhanced dynamic tumor permeability measurement in a MMTV-PyMT tumor. MRI was performed on a 9.4 T horizontal bore system (Varian Inc). Mice were anesthetized with 1.5% isoflurane and body temperature was maintained by a water blanket. Dynamic T1 weighted images were acquired prior- and post- injection Gd-DTPA (Magnevist) over a 40 minute period. A. shows a T_1 weighted spin echo image (TR = 400msec, TE = 10msec) prior to injection with primary tumor sites indicated by the arrows. Spin echo images were used to minimize magnetic susceptibility artifacts that can be prevalent in lower abdominal images at high field strength. Dynamic T_1 changes could be evaluated by the dynamic T_1 images in conjunction to a proton density image obtained prior to the gadolinium injection. Plasma contrast agent concentration was measured in a more anterior imaging slice with larger blood vessels as identified by flow sensitive high flip angle FLASH images. Tumor permeability-surface area product, K (ul/g/min), and blood volume, V (ul/g), were evaluated on a voxel by voxel basis by a Patlek plot of tissue Gd concentration as a function of plasma Gd concentration. B and C show the corresponding maps of K and V.

In collaboration with Luciano Rossetti, we have now learned a more complex catheterization procedure that the Rossetti lab has used

for stable, long term catheterization of mice. It involves catheterization of the jugular vein and enables us to have an implanted catheter that is present for several days. The protocol we now use is the following:

Jugular vein catheterization procedure for MRI

Silastic (0.51 ID) catheter is used to cannulate the right external jugular vein.
The mouse anesthetized by IP injection of Avertin (2.5%).

· To limit the chance of infection, the surgery is performed using a face mask, and the operative field, instruments autoclaved.
· The catheter is filled with heparinized saline (2500 U heparin/250 ml saline).
· A longitudinal incision is made in the skin just over the spot where the anterior jugular, acromiodeltoid, and cephalic veins join together; then, the connective tissue surrounding this spot is carefully removed.
·Three thin threads of suture are passed under the jugular vein below the level of the junction and separated by about 3 mm.
· The cephalic thread is tied to prevent bleeding.
· A small incision is made just below the ligature, and the cannula is pushed into the lumen.
· A few drops of blood are withdrawn to confirm correct catheter placement into the cavum.
· The catheter is then fixed with the threads previously used to tie the jugular vein.
· Backward blood flow from the heart is prevented by loosely tying the jugular vein around the catheter with second and third thread.
· The catheter is then threaded under the skin to the back of the neck and secured with sutures.
· The mouse is kept in a warm place until fully recovered from anesthesia.

A second problem we identified was that rapid imaging of the entire tumor using 125 um x 125 um x 125 um or smaller pixels literally blew fuses on the control apparatus, constraining us to reduce the resolution such that pixels are currently 125um x 125 um x 500 um. This problem was resolved by waiting at least 10 seconds between successive collections.

We then acquired several data sets using the small molecular weight compound Magnevist. Although we were able to acquire images and identify changes in susceptibility due to influx of Magnevist, we were unable to accurately determine the blood input function. Imaging either of the tail or of larger vessels in the images did not provide a consistent time course of the blood levels of Magnevist that could be used to determine the actual dynamics of influx of Magnevist into the tumor. The MRI facility is planning to develop a second specialized tail imaging coil to allow higher resolution imaging of the tail veins in order to accurately determine the dynamics of MRI labels in the blood during the imaging sequence, as has been recently reported by others(1).

We also performed several experiments using a commercially available higher molecular weight compound – BSA with Gadolinium coupled to it. Such compounds should remain present in the blood for a longer period of time, and thus the ability to accurately determine the dynamics of the label in the blood is not as critical. The levels of label were relatively low, and thus we conclude that higher concentrations and labeling ratios are required for successful imaging using this compound.

Task 2. Determine whether regions of increased blood vessel leakiness or increased macrophage density in the primary tumor as identified by MRI correspond to intravasation sites (Months 4 - 8):

a. *Using the methods developed in Task 1, use two-photon microscopy to image sites of increased blood vessel leakiness as determined by MRI for intravasation – 5 mice.*
b. *Using the methods developed in Task 1, use two-photon microscopy to image sites of macrophage density as determined by MRI for intravasation – 5 mice.*
c. *Using the methods developed in Task 1, use two-photon microscopy to image sites of showing both blood vessel leakiness and macrophage density as determined by MRI for intravasation – 5 mice.*

The catheterization problem that slowed Task 1 also slowed our progress for Task 2. However, we were able to develop a solution specifically for two photon imaging by using a needle to pierce the jugular vein, insert a catheter and then seal the insertion site. This procedure leaves the needle near the mouse, and therefore cannot be used for MRI imaging, but the presence of the needle has no effect on two photon imaging. This enabled us to follow the dynamics of Texas Red labeled dextrans (Figures 2 and 3) in preliminary studies.

The results in Figures 2 and 3 show that the permeability properties of blood vessels vary with the size of the probe being used : 3K dextrans are highly permeable, while 70K dextrans are much less permeable. This confirms that we should evaluate

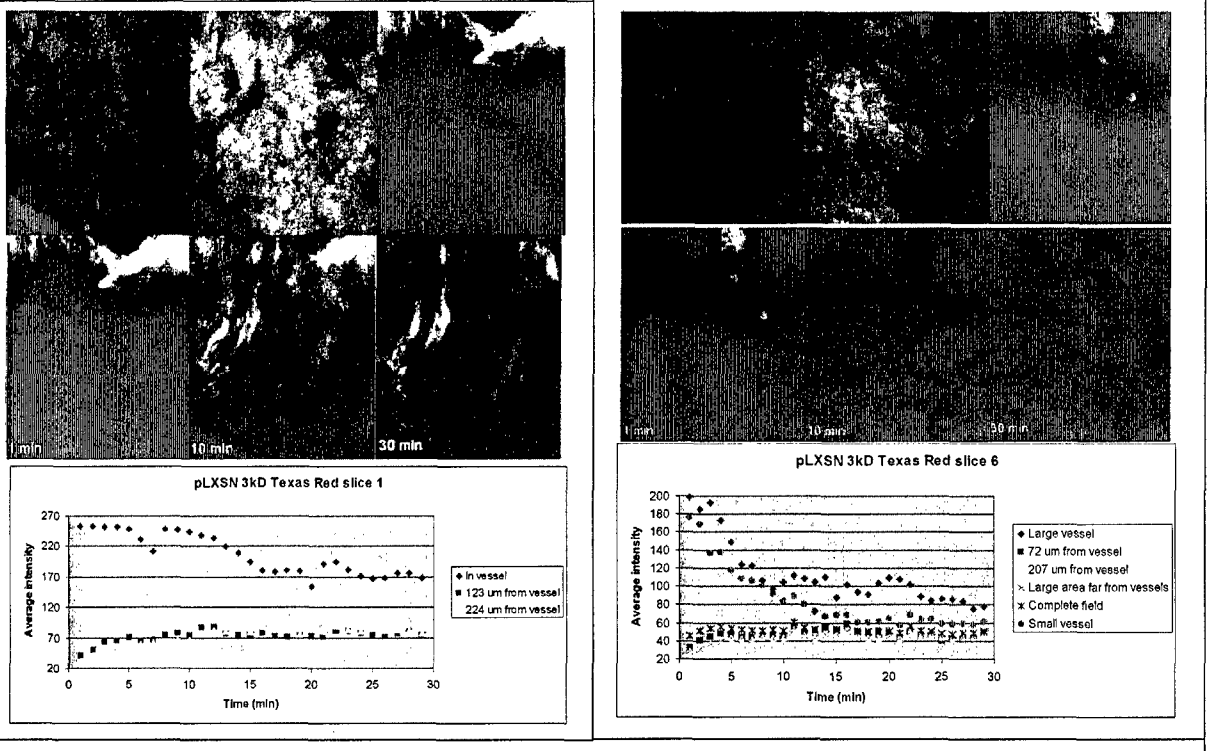

Figure 2. Two photon imaging of blood vessel permeability of 3kD Texas Red dextran (at 0 min) in MTLn3-pLXSN tumor at 10 um (slice 1) and 60 um (slice 6) into the tumor. There was no signal in the red channel before injection, and time lapse zeries were collected every 10 um for 100 um into the tumor every minute after injection of the dye. For each side, the top row shows merged image, tumor and blood, second row is blood at 1, 10 and 30 min post injection, and bottom row shows quantitation of vessel intensity and various areas outside the vessels.

different sized MRI probes in order to determine which probe might best correlate with metastatic ability.

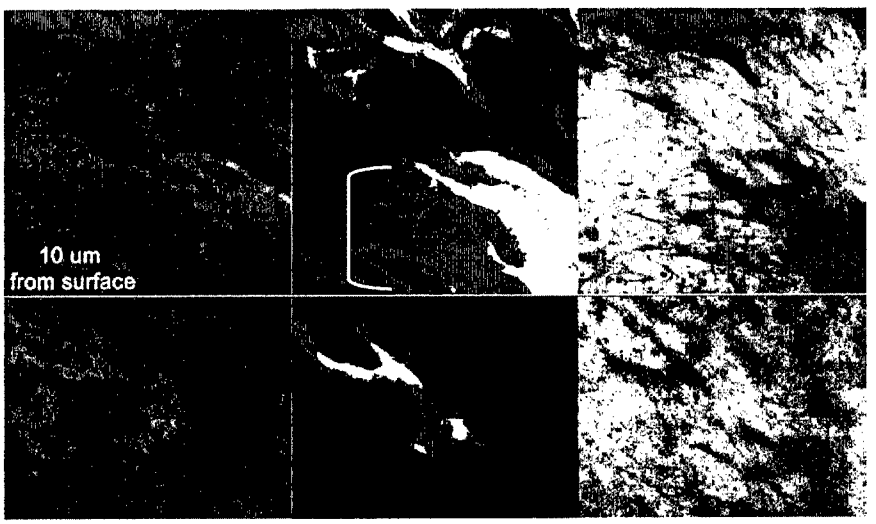

Figure 3. Intravital Imaging of 70 kD Texas Red dextran 1 hour after iv injection in MTLn3-pLXSN GFP tumor. Top image shows dextran in blood vessels (center), tumor cells (right) and merged images (left) near the tumor surface (top) and 60 um deeper (bottom). The bracket in the top image marks region of extravasated dextran in the top image.

We have developed two methods for two photon imaging of macrophages for correlation with MRI. One is to use phagocytosis of preinjected high molecular weight Texas Red labeled dextrans (Figure 4). The other method utilizes expression of GFP in macrophages, but will not be useful until we develop a separate label for tumor cells (we are generating transgenic animals that will express CFP in the tumor cells). This will enable use to track macrophages, tumor cells, and blood vessel leakiness in 3 separate channels.

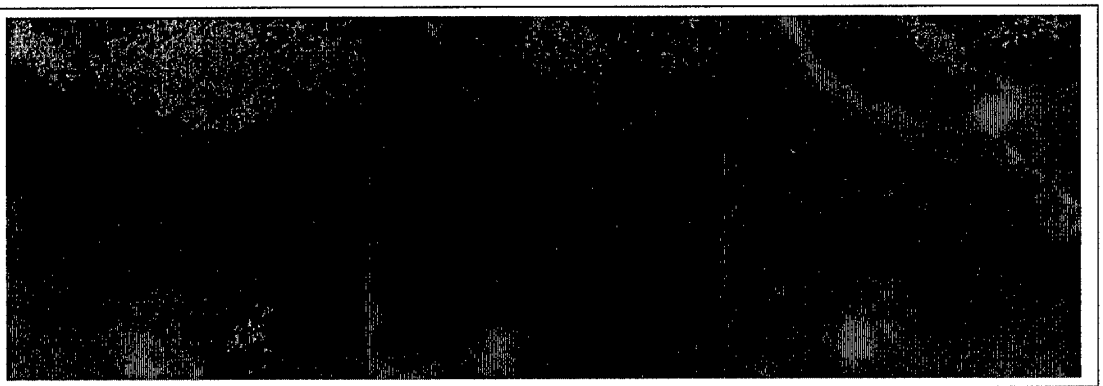

Figure 4: Two photon imaging of macrophages (red) around PyMT transgenic tumors expressing GFP (green) near surface (left), 50 um (middle) and 100 um (right) into the tumor. Macrophages were labeled by tail vein injection of 70K Texas Red dextran 1 – 2 hours before imaging

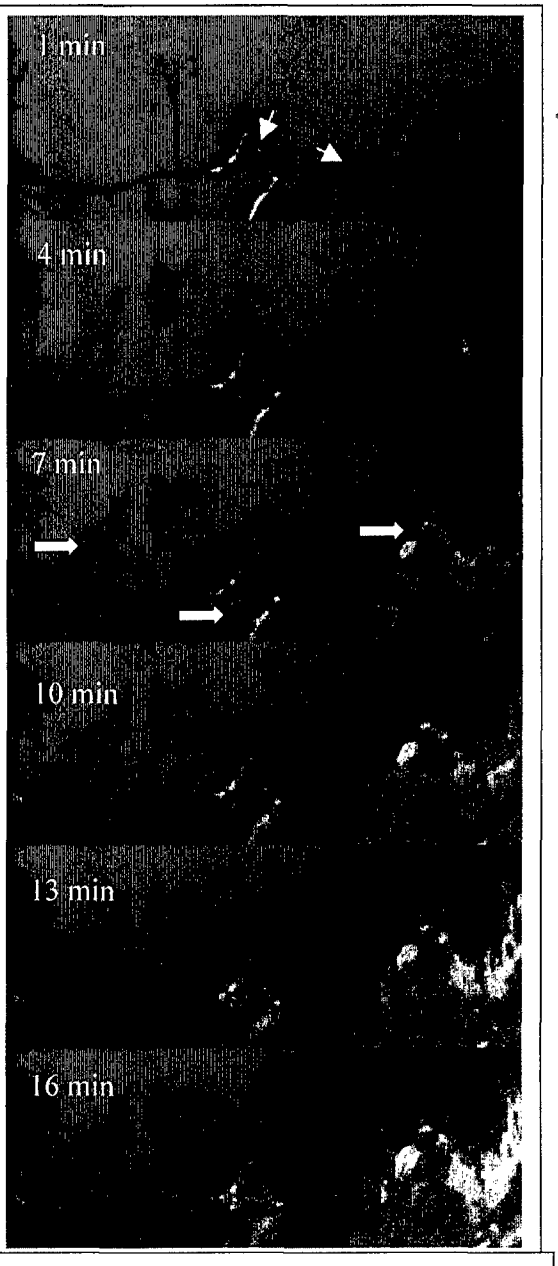

Figure 5. Heterogeneous filling dynamics of tumor blood vessels. Under anesthesia the surface of the tumor was exposed and imaged using a fluorescence dissecting microscope. Images were taken at 3 minute intervals starting at 1 minute after injection of Rhodamine B. Some vessels are already filled by 1 min (small arrows in the 1 min frame), but then other vessels slowly fill with label over the next 5 – 10 min (large arrows in 7 min frame identify 3 examples).

In the course of these studies we discovered another issue that casts serious doubt on the interpretability of MRI imaging of tumors using Magnevist for dynamic permeability studies. Upon using fluorescent dissecting microscopy time lapse imaging to follow the flow of rhodamine into the tumor upon iv injection, we found that the influx of the dye into tumor vessels was extremely heterogeneous and slower than the influx into normal tissues (Figure 5). This is consistent with scattered reports in the literature that tumor vasculature has irregular flow (e.g., (2)), but has serious implications for using time resolved MRI permeability measurements (e.g., (3)). One of the assumptions made for measuring permeability is that the level of Magnevist in the tumor vasculature is that same as the level in other normal vessels in the animal. We have found that this assumption is incorrect for our tumor models, and is likely to be incorrect for others as well. Delayed dynamics which are interpreted as increased permeability could actually reflect intermittent blood flow in tumor vessels rather than leak from the vessels. This again supports the argument that studies of permeability and vascularity may be more reliable using higher molecular weight compounds which are not so rapidly cleared from the circulation.

Task 3. Compare MRI measurements of the poorly invasive, macrophage deficient, op/op pyMT tumor to invasive wild-type pyMT tumors to determine whether increases in vessel leakiness and/or macrophage density in the wild-type pyMT model as measured by MRI can be used to identify the invasive stage (Months 8 - 12):

a. *Measure blood vessel leakiness and macrophage density in*

wild-type pyMT tumors using MRI during the transition from noninvasive adenoma to invasive carcinoma – 5 mice followed every 3 weeks until week 18.

b. *Measure blood vessel leakiness and macrophage density in op/op pyMT tumors using MRI and compare with wild-type pyMT tumors –5 mice followed every 3 weeks until week 18.*
c. *After final measurement, perform light microscopy imaging to determine whether intravasation sites correlate regions of increased leakiness or blood vessel density in the wild-type pyMT tumors – the 10 mice imaged in 3a,b.*

As shown in the figures above, we have been using wild-type PyMT tumors for a number of initial measurements. However, we have not begun studying the time course of this transition nor how the op/op PyMT tumors differ from wild-type due to the technical problems described above with MRI imaging.

KEY RESEARCH ACCOMPLISHMENTS

- We have established expertise in jugular vein catheterization suitable for MRI imaging
- We found that heterogeneous filling kinetics of tumor blood vessels casts doubt on the validity of using Magnevist for tumor permeability studies, and supports the use of higher molecular weight labels
- We have established methods for labeling macrophages for two photon imaging
- We have established methods for evaluating blood vessel leakiness using two photon imaging.

REPORTABLE OUTCOMES

- Submission of grant proposal to the NIH for further studies of tumors and intravasation using MRI.

CONCLUSIONS

We have acquired expertise in catheterization and have developed imaging sequences for studying PyMT and xenograft mammary fat pad tumors using a 9.4T MRI system. Methods for high resolution imaging of the tail vein together with analysis using higher molecular weight compounds are ongoing. We have identified heterogenous tumor vessel filling dynamics as a possible confounding artifact in the analysis of tumor vessel permeability using MRI.

We have established methods for labeling macrophages in order to compare sites of macrophage density with vessel leakiness in two photon imaging. In addition, we have established protocols for evaluating tumor vessel permeability in two photon imaging relative to tumor cell motility. These techniques will improve our ability to accurately compare blood vessel permeability and macrophage density for tumor models of varying metastatic ability.

We have submitted an R01 proposal that includes the data presented here as preliminary data showing feasibility for studies that we propose to perform that will pursue the aims described above.

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APPENDICES:

Personnel supported by this grant:

1. Richard Kennan (MRI collaborator)
2. Jeffrey E. Segall, P.I.

Total Publications:

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tumor cell motility *in vivo* coordinately with enhanced intravasation and metastasis. Cancer Res.
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14. Wyckoff, Wang, Lin, Stanley, Segall, Pollard, and Condeelis (2005) Direct visualization of macrophage mediated intravasation of carcinoma cells in mouse mammary tumors. (Submitted).